Original Article

Protection of Hesperidin against Methotrexate-Induced Nephrotoxicity may be Mediated by Nrf2/HO-1 Pathway

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ABSTRACT

Background: Methotrexate (MTX), a successfully used chemotherapeutic in the treatment of various malignancies and autoimmune diseases, might cause severe nephrotoxicity. Here, we aimed at investigating possible nephroprotective effects of hesperidin (HES), a flavanone present in citrus fruits, against MTX-induced toxicity. **Methods:** Rats were divided into control, HES, MTX, and MTX/HES groups, where HES was administered in a dose of 100 mg/kg/day orally for 8 days and MTX in a single i.p. dose of 20 mg/kg on day 5 of the experiment. **Results:** Pretreatment with HES significantly improved MTX-induced deteriorated kidney function and structure, as well as reversed MTX effects on renal tumor necrosis factor (TNF)-α level and caspase 3 expression. MTX upregulated renal breast cancer resistance protein (BCRP); an efflux transporter that extrudes MTX from the kidney. Unfortunately, MTX/HES did not show a further increase in BCRP expression but rather showed downregulation. MTX also caused downregulation of renal nuclear factor erythroid 2-related factor 2 (Nrf2) and hemeoxygenase-1 (HO-1) expressions, whereas HES reversed the MTX effect and upregulated renal Nrf2/HO-1. **Conclusion:** HES conferred protection against MTX-mediated nephrotoxicity, at least in part via anti-inflammatory and anti-apoptotic mechanisms. Nrf2/HO-1 pathway, but not BCRP, may have a role in HES-induced nephroprotection against MTX toxicity.

Key words: Methotrexate, Hesperidin, Nrf2, HO-1, TNF-α, Caspase 3, BCRP.

INTRODUCTION

For more than six decades, methotrexate (MTX) has been successfully used in a high dose for the treatment of various types of cancers[1] and in a relatively smaller dose to control autoimmune diseases.[2] Unfortunately, MTX, especially at high dosages, might cause severe acute renal toxicity in up to 12 % of patients.[3] The mechanism of MTX-induced nephrotoxicity is not yet fully understood. However, it has been suggested that MTX, especially when given in high dose, may cause glomerular and tubular dysfunction that might lead to the delay of its own elimination and cause its precipitation in high concentrations in renal tubular cells, causing their obstruction and eventually acute renal injury.[4] Many protein transporters govern the uptake of MTX from the blood through the renal brush border membrane into proximal tubule cells and its subsequent efflux through the apical membrane into the urine.[5] Breast cancer resistance protein (BCRP) is one of the main renal apical efflux transporters that accept MTX as a substrate.[6] We have previously shown that MTX-induced renal toxicity might be reversed by resveratrol, at least in part, through modulation of BCRP

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expression in rats *in vivo*.[7] *In vitro* studies also showed that uremic toxins produced during renal damage might further inhibit transport of MTX by BCRP,[8] thus entering in a vicious circuit.

Hesperidin (3,5,7-trihydroxyflavanone 7-rhamnoglucoside; HES), the glycosylation product of hesperitin (40-methoxy-30,5,7-trihydroxyflavanone) dietary present almost exclusively in citrus fruits, tomatoes, and some aromatic plants as mint, is reported to have health-promoting effects that may be beneficial in various diseases as hypertension, myocardial infarction, diabetes, neurological disorders, and cancer.[9] HES is commercially available under different trade names, mostly as a drug combination with the flavonoid diosmin in different ratios, ranging from 1:9 to almost 1:1. Due to its phlebotonic effects, these drug combinations show beneficial effects in improving vascular tone, supporting lymphatic drainage, and strengthening vascular structure; thus, they are indicated for the treatment of acute hemorrhoid, varicose veins, and chronic venous disease. In addition, HES also showed a protective effect against hepatotoxicity induced by another anticancer drug, cisplatin, without affecting its anticancer efficacy.[10] In the kidney, HES has shown protective effects against renal toxicity caused by 5-fluorouracil,[11] cyclophosphamide,[12] sodium arsenite,[13] acrylamide,[14] gentamicin,[15] and iron.[16] The aim of the current study is to investigate possible nephroprotective effects of HES on MTX-induced toxicity in rats. In addition, we investigate the involvement of BCRP transporter and/or nuclear factor erythroid 2-related factor 2 (Nrf2)/hemeoxygenase-1 (HO-1) pathways as means of HES-induced protection against MTX-induced nephrotoxicity.

MATERIALS AND METHODS

Drugs and Chemicals

HES and MTX (25 mg/ml ampoules) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Ebewe Co. (Unterach, Austria), respectively. Kits for assessment of blood urea nitrogen (BUN) and creatinine were purchased from Spectrum Diagnostics (Cairo, Egypt). Quantikine rat tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals were obtained from commercial sources and of analytical grade. RNeasy Purification Reagent and QuantiTect Reverse Transcription Kit were purchased from Qiagen (Valencia, CA, USA). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA).

Animal Study Protocol

Twenty-four male Wistar rats weighing 190 ± 10 g were left to acclimatize for one week before the start of experiments. Rats were housed under standard laboratory animal conditions, at 24 ± 2°C, freely accessing standard laboratory animal chow and tap water. Animal protocol ethically followed the Research Ethics Committee guidelines, King Faisal University, which is in accordance with the National Committee of BioEthics (NCBE), KACST, Riyadh, Saudi Arabia. Animals were divided into four groups (n=6). The first group served as control. The second group was treated with freshly prepared HES powder in 0.5 % carboxymethyl cellulose in a dose of 100 mg/kg/day orally[12,13] for 8 days. The third group was treated with a single intraperitoneal injection of MTX in a dose of 20 mg/kg[7] on day 5 of the experiment. The fourth group received both HES and MTX in combination at the previously indicated dosage regimens.

Sampling Procedures

After the 8th day of the experiment, animals were sacrificed. Blood samples were collected and centrifuged at 3000 g for 15 min to obtain the sera that were aliquoted and stored at −20°C till used. Both kidneys were rapidly extracted, and a longitudinal slice was taken from the right kidney of each animal for histopathological examination. The cortices of the remainder of both kidneys were snap-frozen in liquid nitrogen and kept at −80°C. Kidney tissues were homogenized in 20% w/v ice-cold phosphate buffer (0.01 M, pH 7.4). The homogenate was centrifuged at 3000 g for 15 min at 4°C, and the supernatant was obtained, then divided over a number of containers to avoid sample thawing and refreezing and kept at −80°C until used.

Renal Histopathological and Biochemical Assessment

Longitudinal slice from each rat kidney was fixed in 10% neutral buffered formalin solution. Samples were then dehydrated in gradual ethanol (70–100%) and cleared in xylene. Afterward, tissue samples were embedded in paraffin. Sections of paraffin blocks of 5 µm thickness were cut by microtome and mounted on clean glass slides. Staining of the slides was performed using regular hematoxylin and eosin stain for histopathological analysis under a light microscope. Scoring of pathological changes was performed and was qualified as mild, moderate, or severe if less than 25, 50, and 75% of the total fields tested were distorted, respectively.[17] As a marker of renal function and nephrotoxicity, biochemical assessment of serum BUN and creatinine using commercially available

Table 1: Sequences of rat primers used in real-time polymerase chain reaction determined using Genbank database.

	Type of primer	Primers	GenBank accession number	Product size
Caspase-3	Forward	5'-GGAGTCTGACTGGAAAGCCG-3'	NM_012922.2	192 bp
	Reverse	5'-ACCCGCCCCTTGAATTTCTC-3'		
BCRP	Forward	5'-TGTAGGTCGGTGTGCGAGT-3'	NM_181381.2	240 bp
	Reverse	5'-CCGGACTAGAAAGCCACTCT-3'		
Nrf2	Forward	5'-TTGCCGCTCAGAACTGTAGG-3	NM_031789.2	245 bp
	Reverse	5'-GTTGCCATCTCTGGTCTGCT-3'		
HO-1	Forward	5'-ACCTACCTCTGCAAAGACGC-3'	NM_012580.2	149 bp
	Reverse	5'-GGGGCCAACACTGCATTTAC-3'		
β-actin	Forward	5'-TATCCTGGCCTCACTGTCCA-3'	NM_031144.3	130 bp
	Reverse	5'-AAGGGTGTAAAACGCAGCTCA-3'		

BCRB: Breast cancer resistance protein; Nrf2: Nuclear factor erythroid 2-related factor 2; HO-1: Hemeoxygenase-1.

kits was performed colorimetrically, according to the manufacturer's guidelines.

Assessment of TNF-α in Renal Homogenate Using ELISA Kit

The level of renal TNF-α was evaluated according to manufacturer's instructions, where 50 µl of kidney homogenate was added to 50 µl solution of sample diluent and put in a 96-wells ELISA plate, with 2 wells containing sample diluent only without tissue homogenate to serve as blank. After mixing, the plate was incubated for 2 hr at room temperature as a first incubation. Afterward, the ELISA plate was washed 5 times using 400 µl of diluted washing buffer, followed by drying. An amount of 100 µl of rat TNF-α conjugate was dispensed in each well, except for the blank wells, then incubated again and washed as previously described. From each substrate solution, 100 µl were added to each well; then the plate was covered to protect from light and incubated for 30 min at room temperature. The reaction was terminated by adding a 100 µl stop solution. Using an ELISA plate reader, the plate was read at 450 nm, and the concentration was evaluated via the standard curve.

Real-Time Polymerase Chain Reaction (PCR)

For assessment of expression of caspase 3, BCRP, Nrf2, and HO-1, real-time PCR was carried out. Using RNeasy purification reagent, total RNA was isolated from kidney tissue homogenate according to the manufacturer's instructions. Denaturation of total renal RNA was performed at 70°C for 2 min, which was then reverse-transcribed using QuantiTect Reverse Transcription Kit. The real-time PCR procedures were done using SYBR Green PCR Master Mix together with 200 ng of each primer. The sequences of the forward and reverse primers of caspase 3, BCRP, Nrf2, and HO-1, as well as β-actin, are listed in Table 1. PCR reactions comprised of 1 cycle at 95°C for 10 min, 94°C for 15 s, and 40 cycles at 60°C for 1 min, which were performed on a StepOnePlus System (Applied Biosystems). Analysis of data was performed employing ABI Prism 7500 Sequence Detection System software, and quantification was done via version 1.7 Sequence Detection Software from PE Biosystems (Foster City, CA, USA). Normalization of the expression of the studied gene was performed relative to β-actin utilized as an internal standard, and calculation was performed using the comparative threshold cycle method.[18]

Statistical Analysis

Data were presented as means ± SEM, and statistical analysis was performed by GraphPad Prism version 5.00 for Windows (San Diego, CA, USA), via Tukey-Kramer post-analysis test that compares all groups, preceded by one-way analysis of variance (ANOVA). The *P* value was considered significant if less than 0.05.

RESULTS

Effect of HES on Renal Histopathological Changes

Renal histopathological changes are presented in Figure 1. Both control and the group treated orally with 100 mg/kg/day of HES for 8 days showed normal glomerular and tubular renal architecture (Figure 1A,B). On the other hand, a single i.p. dose of MTX of 20 mg/kg caused apparent renal picture distortion (Figure 1C). Scoring of different pathological features showed that the MTX group had mild tubular necrosis, with

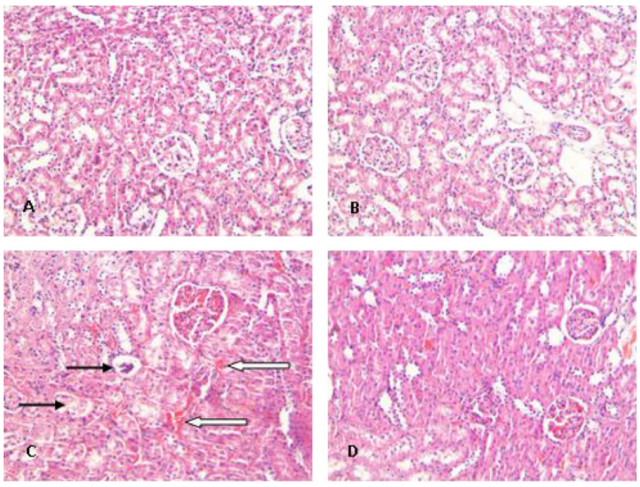

Figure 1: Effect of hesperidin (HES) on histopathological changes of rat kidney in methotrexate (MTX)-induced toxicity. Rat kidney photomicrographs stained by hematoxylin and eosin (×200) of (A) control, (B) HES, (C) MTX and (D) MTX/HES groups. Black arrow, pyknotic distorted glomeruli; white arrow, blood vessel congestion and extravasations. Scoring of histopathological changes is summarized in Table 2.

Table 2: Effect of hesperidin (HES) on histopathological scoring of microscopic renal picture in methotrexate (MTX)-induced nephrotoxicity in rats.

	Tubular necrosis	Glomerular damage	Congested blood vessels	Leucocytic infiltration
Control	-	-	-	-
HES	-	-	-	-
MTX	+	++	+++	++
MTX/HES	-	-	+	+

The (-) was considered normal histological findings. The (+), (++) and (+++) were indicative of mild, moderate and severe histological distortions of less than 25, 50 and 75% in total fields tested, respectively. Results are of sections of renal tissues from each animal (n = 6), 3 fields/section.

moderate glomerular damage and leucocytic infiltration, accompanied by severely congested blood vessels (Table 2). Pretreatment with HES prior to MTX challenge reversed glomerular and tubular damage to normal picture (Figure 1D), with only mild congested blood vessels and minimal leucocytic infiltrations.

Effect of HES on Renal Function Parameters

To test kidney function, BUN and creatinine were biochemically assessed in serum. MTX caused a significant increase of both BUN and creatinine levels compared to control (Figure 2A,B). Pretreatment of HES in the MTX-challenged group caused a significant decrease in both BUN and creatinine compared to MTX alone.

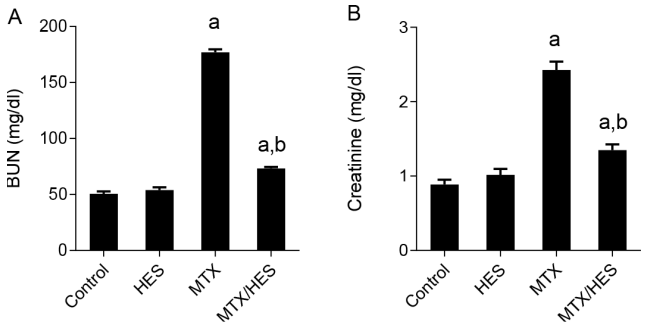

Figure 2: Effect of hesperidin (HES) on blood urea nitrogen (BUN) and creatinine in methotrexate (MTX)-treated rats. Values represent means ± SEM (n = 6). Significant difference is reported when P is less than 0.05. "a" Significant difference compared to control and "b" significant difference compared with MTX group.

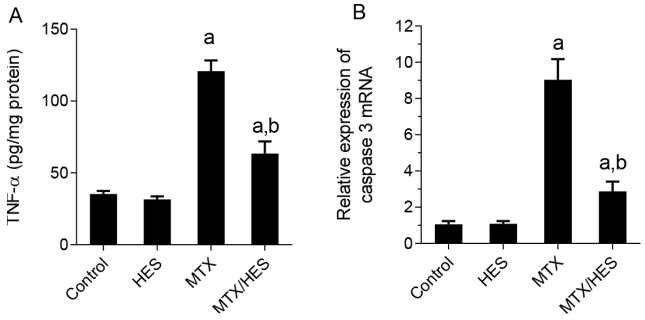

Figure 3: Effect of hesperidin (HES) on renal level of tumor necrosis factor-α (TNF-α) and caspase 3 mRNA expression in methotrexate (MTX)-treated rats. (A) Renal level of TNF-α assessed by enzyme-linked immunosorbent assay (ELISA) and (B) caspase 3 mRNA relative expression to β-actin assessed by real-time polymerase chain reaction. Values represent means ± SEM (n = 6). Significant difference is reported when P is less than 0.05. "a" Significant difference compared with control and "b" significant difference compared with MTX group.

Effect of HES on Renal Inflammatory and Apoptotic Markers

MTX caused a significant increase in the renal level of TNF-α compared to control (Figure 3A). The group receiving both MTX and HES, however, showed a decrease in renal tissue TNF-α compared to the group receiving MTX alone. Similarly, the pro-apoptotic marker caspase 3, determined via real-time PCR, in the MTX-treated group showed a significant increase in renal expression compared to control (Figure 3B). On the other hand, MTX/HES concomitantly treated group showed a decrease in renal expression of caspase 3 compared to the sole MTX-treated group.

Effect of HES on Renal BCRP, Nrf2, and HO-1 mRNA Expression

Expression of BCRP in the kidney was not affected by sole treatment with HES at an oral dose of 100 mg/kg

for 8 days (Figure 4). However, MTX-treated rats showed significantly increased expression of renal BCRP compared to control. Pretreatment of MTX-challenged rats with HES, however, caused a significant reduction of BCRP expression compared to the MTX-treated group. Expression of both Nrf2 and HO-1 was not affected by sole treatment of HES compared to control (Figure 5). MTX treatment, on the other hand, caused a significant decrease in renal expression of both Nrf2 and HO-1 compared to control. Treatment with HES in MTX-challenged rats, however, caused a significant increase of both Nrf2 and HO-1 compared to MTX alone, to levels insignificant from that of control.

DISCUSSION

In the current study, MTX caused nephrotoxicity, as evident by significantly increasing kidney function tests and disrupting normal renal histological architecture, which is in line with our previous studies.[7,19,20] To investigate the mechanisms by which MTX may induce nephrotoxicity, the current study showed that MTX caused an increase in renal levels of TNF-α and caspase 3, indicating the stimulation of inflammatory and apoptotic pathways as possible mechanisms of MTX-induced nephrotoxicity, which is also in agreement with previous results.[19-21]

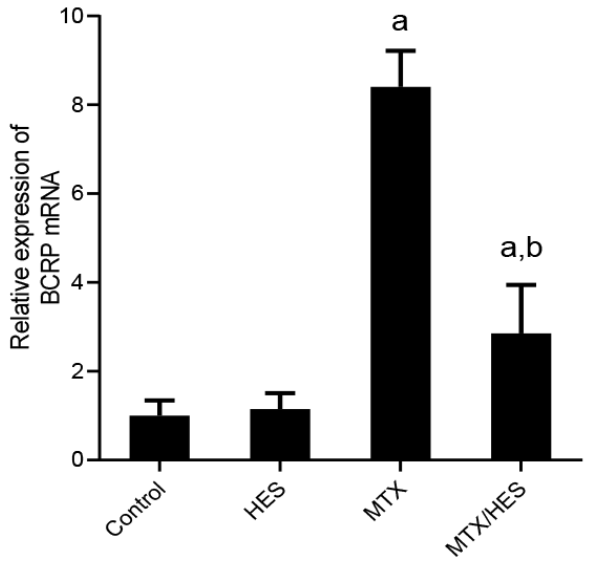

Figure 4: Effect of hesperidin (HES) on renal level of breast cancer resistance protein (BCRP) mRNA expression in methotrexate (MTX)-treated rats. BCRP mRNA relative expression to β-actin was assessed by real-time polymerase chain reaction. Values represent means ± SEM (*n* = 6). Significant difference is reported when *P* is less than 0.05. "a" Significant difference compared with control and "b" significant difference compared with MTX group.

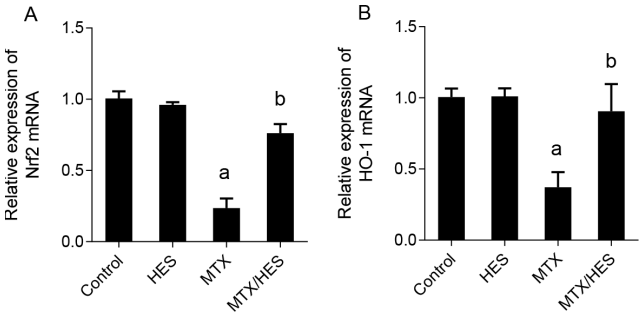

Figure 5: Effect of hesperidin (HES) on renal mRNA expression of (A) nuclear factor erythroid 2-related factor 2 (Nrf2) and (B) hemoxygenase-1 (HO-1) in methotrexate (MTX)-treated rats. The mRNA relative expression to β-actin assessed by real-time polymerase chain reaction. Values represent means ± SEM (n = 6). Significant difference is reported when *P* is less than 0.05. "a" Significant difference compared with control and "b" significant difference compared with MTX group.

Pretreatment with HES improved renal function in MTX-treated rats, as evident by the significant decrease in BUN and creatinine. Consistent with our results, several previous studies showed that HES improved renal function impairment caused by 5-fluorouracil,[11] cyclophosphamide,[12] sodium arsenite,[13] acrylamide,[14] gentamicin,[15] and iron.[16] In addition, pretreatment with HES tremendously improved renal pathological distortions induced by MTX. Similar histopathological findings of HES-induced nephroprotection were reported in renal damage induced by 5-fluorouracil,[11] cyclophosphamide,[12] sodium arsenite,[13] gentamicin,[15] and cisplatin.[22]

In the current study, HES showed anti-inflammatory properties, as it ameliorated the increased TNF-α level induced by MTX in the kidney. Previous studies showed that HES may possess a similar anti-inflammatory effect and may decrease renal tissue levels of TNF-α induced by cyclophosphamide,[12] sodium arsenite,[13] gentamicin,[15] and sodium fluoride.[23] Besides the kidney, HES was also reported to have a comparable anti-inflammatory effect in other organs like the liver, as it decreased TNF-α in sodium arsenite[13] and sodium fluoride[23] induced hepatotoxicity. In addition, HES decreased TNF-α in sodium arsenite-induced cardiotoxicity and neurotoxicity.[24] TNF-α binds to its receptors and stimulates receptor-dependent pathway of apoptosis through activation of caspases, including caspase 3.[25] Thus, in addition to its anti-inflammatory effects, HES, in the current study, showed an anti-apoptotic effect, as it decreased the renal expression of the pro-apoptotic marker; caspase 3, induced by MTX. Comparable results on caspase 3 were previously documented in previous studies, where HES had an anti-apoptotic

effect in nephrotoxicity induced by 5-fluorouracil,[11] cyclophosphamide,[12] sodium arsenite,[13] cisplatin.[22] and trichloroethylene.[26] Other than the kidney, HES was also reported to decrease hepatic caspase 3 induced by sodium arsenite[13] and carbon tetrachloride.[27] In addition, HES diminished caspase 3 in sodium arsenite-induced cardiotoxicity and neurotoxicity.[24] These multi-organ results support that HES might have generalized multi-organ anti-inflammatory and anti-apoptotic properties.

In the current study, MTX caused more than an 8-fold increase in renal expression of BCRP, which is in line with our previous results.[7] Interestingly, HES in the present study, instead of increasing renal BCRP, rather, it decreased it, suggesting no role of BCRP in HES-induced nephroprotection against MTX. Still, in the kidney, MTX is accepted as a substrate by a number of apical transporters, other than BCRP, that actively efflux MTX at the expense of ATP, including a number of multidrug resistance proteins.[5,28] It is possible that HES might have mediated its nephroprotective effects by affecting the expression of other transporters capable of modulating MTX renal excretion, which is mere speculation in need of further investigations.

Nrf2/HO-1 pathway is one of the main mechanisms that combat oxidative stress. As an upper stream regulator, Nrf2 binds HO-1 antioxidant responsive element, initiating a plethora of genetic transcriptional activation that results in the production of antioxidant and cellular protective proteins that eventually ameliorate oxidative stress.[29] In the present study, MTX downregulated the expression of both Nrf2 and HO-1 in rat kidneys. Previous studies showed similar MTX downregulation of Nrf2/HO-1 pathway in kidney[30,31] In the current study, HES reversed the down regulatory effect of MTX and caused upregulation of both Nrf2 and HO-1 renal expression. Interestingly, in the current study, HES alone without MTX challenge had no effect on either renal Nrf2 or HO-1 expression. The reported effects of HES on the Nrf2/HO-1 pathway are controversial. In the kidney, HES, with or without challenge, was reported to upregulate renal Nrf2 and/or HO-1 in a model of diethylnitrosamine/carbon tetrachloride-induced kidney repercussions.[32] On the other hand, only HES+gentamicin, not HES alone, could induce the expression of Nrf2 and/or HO-1 in gentamicin-induced nephrotoxicity model.[15]

It is possible that HES-induced upregulation of antioxidant Nrf2/HO-1 pathway in MTX-challenged rat kidney in the current study had triggered the amelioration of inflammation and/or apoptosis, depicted by the decrease in renal levels of TNF-α and caspase 3 expression. We have previously shown that the modulation of HO-1 expression and/or activity may affect renal levels of TNF-α and caspase 3,[33] suggesting that their upregulation by MTX possess an upper stream factor that may govern renal inflammation and apoptosis, thus inducing nephrotoxicity. The cross-talk between these pathways has indeed been previously reported, where upregulation of renal Nrf2/HO-1 was concomitantly encountered together with a decrease in renal TNF-α by ergothioneine in cisplatin-induced nephrotoxicity,[34] by phenethyl isothiocyanate in streptozotocin-induced diabetic nephropathy,[35] and by quercetin on copper sulfate-induced nephrotoxicity.[36] Similar findings have been reported linking the upregulation of renal Nrf2/HO-1 to caspase 3 downregulation by ergothioneine in cisplatin-induced nephrotoxicity,[34] by camel milk in cyclosporine-induced nephrotoxicity,[37] and by thymoquinone in diclofenac-induced acute kidney injury.[38] However, despite all previous reports, it is extremely difficult to hypothesize a cause-effect relationship between these complex pathways.[39]

CONCLUSION

Despite not having any favorable effects on renal BCRP expression, still HES pretreatment succeeded in ameliorating MTX-induced nephrotoxicity. The mechanisms involved included, at least in part, reversing renal inflammatory and apoptotic status deteriorated by MTX. In addition, HES reversed renal downregulation of Nrf2/HO-1 expression by MTX.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BCRP: Breast cancer resistance protein; **BUN:** Blood urea nitrogen; **ELISA:** Enzyme-linked immunosorbent assay; **HES:** Hesperidin; **HO-1:** Hemeoxygenase-1; **MTX:** Methotrexate; **Nrf2:** Nuclear factor erythroid 2-related factor 2; **PCR:** Polymerase chain reaction; **TNF-α:** Tumor necrosis factor-α.

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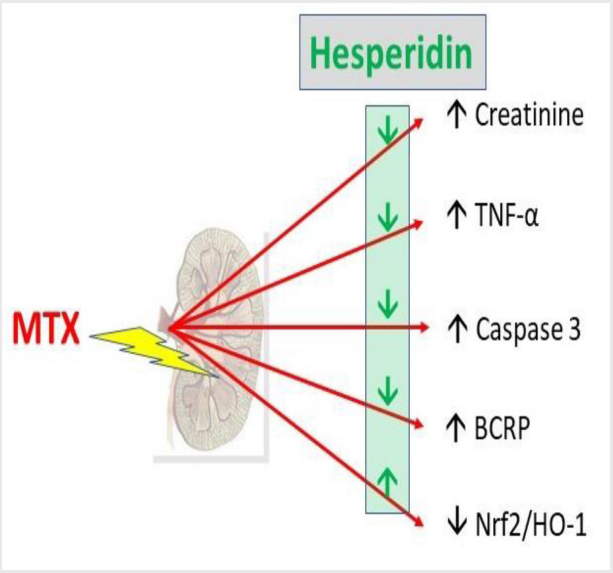
PICTORIAL ABSTRACT



SUMMARY

Hesperidin protects against methotrexate-induced nephrotoxicity. Mechanisms involved is down-regulation of TNF-α and caspase 3. Methotrexate induced renal BCRP expression. Concomitant hesperidin didn't further induce, but inhibited renal BCRP expression. Hesperidin induced Nrf2/HO-1 pathway in methotrexate-induced nephrotoxicity.

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